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EXTERNAL FACTORS, PRODUCED BY GROWING NERVES, TRIGGER A REGENERATIVE RESPONSE IN A NON-REGENERATIVE CENTRAL NERVOUS SYSTEM: PURIFICATION AND MODE OF ACTION

ANNUAL REPORT

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FOREWORD

In conducting the research described in this report, the investigators adhere to the "Guide for the care and use of laboratory animals" prepared by the committee on Care and Use of laboratory Animal Resources, National Research Council (DHEW publication No (NIH)(86-23 Revised 1985).

ABBREVIATIONS

apo-A-I - apolipoprotein-A-I

apo-E - apolipoprotein-E

CM - conditioned media

CNS - central nervous system

FCS - fetal calf serum

FGF - fibroblast growth factor

He-Ne - helium-neon

NGF - nerve growth factor

PNS - peripheral nervous system

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Mammalian central nervous system (CNS) neurons have a negligible post-traumatic regenerative capacity while the nerves of lower vertebrates, as well as the peripheral nervous system(PNS) of mammals regenerate spontaneously after injury. Studies carried out in our laboratory as well as in several other laboratories suggested that CNS neurons may have the intrinsic capacity to regenerate but fail to do so because they are surrounded by an inappropriate or inhospitable environment. We modulated the environment by the application of soluble substances originating from growing nerves (fish optic nerves regenerating after trauma or developing, neonatal rabbit optic nerves) in the form of media conditioned (CM) by such nerves implanted into injured optic nerves of adult rabbits. Last year we identified two components within these CM, apolipoprotein-A-I involving in scavanging lipid degradation products and a glial modulating factor(s) which effects glial cell behavior, as manifested by laminin production, thereby possibly circumventing one of the barriers for regeneration.

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1. Statement of the problem

1.1 The general problem and the significance of this research

The nerves of the mammalian central nervous system very rarely repair themselves after injuries which comprise a considerable proportion of combat casualties and are the most common cause of severe permanent post-traumatic neuromotor paralysis. These injuries, which include brain, spinal cord and major sense organ trauma, cause permanent disability because the adult mammalian central nervous system (CNS) is incapable of functional regeneration. Due to the poor regenerative capacity of mammalian CNS, an injury in the lower spinal cord often results in the permanent confinment of the casualty to a wheel chair. Laceration in the upper spinal cord leads to paralysis of the four limbs and a cut in the optic nerve leads to complete incurable blindness. Furthermore, the inability of the central nervous system to regenerate is the main reason why reperative and transplantation surgery in the CNS can not be performed.

Means which will induce central nervous system regeneration will be of immense benefit to soldiers suffering from these common combat injuries of the head, spinal cord and major sense organs.

1.2 The basic research problem

The process of axonal regeneration involves (i) establishment of a neuronal growth state and formation of new sprouts, (ii) elongation of new sprouts, and (iii) target recognition, synaptogenesis and cessation of axonal elongation. Successful regeneration probably depends on a synchronized progression of the events that are involved in these major phases. Malfunction of any of these events or lack of synchrony may hamper or inhibit the process of regeneration.

Our studies as well as studies carried out in other laboratories provide evidence that the nature of the environment of the injured nerves has a key role in regeneration. Our studies on regeneration have, therefore been focused on identifying the environmental elements which are involved in the response to injury (cellular, soluble and extracellular non-soluble). The information from these studies should provide ways to devise various means of intervention after CNS injury to faciliate regeneration such as: (1) supply of neurotrophic factors which may save neurons from secondary cell death or which activate neurons to grow (2) provide glial cells with growth factors which may modulate their response to the injury and thereby allow them to acquire growth supportive features, such as production of laminin or other substances needed for growth (3) identification of other growth modulating factors such as proteases inhibitors or lipid mediators and (4) search for a combined treatment.

2. Background

Recent studies carried out in various laboratories including our laboratory provide evidence that the environment which surround the injured nerve has a significant influence on determining the regenerating capacity of the nerve.

The emphasis in our study of regeneration of CNS neurons is therefore focused on identifying the factors which allow and induce the neurons to regenerate and on attempting to devise ways to circumvent the impediments to central nerve regrowth.

2.1 Environmental elements and regeneration.

As a result of injury, the neuron is deprived from target-derived substances and its mutual relationship with the environment is disrupted. The state of growth of the surrounding non-neuronal cells is altered.

(Nathaniel & Nathaniel, 1973; Nathaniel & Nathaniel, 1981; Nathaniel & Pease, 1963; Neumann et al., 1983a; Stevenson & Yoon, 1978). This may lead to formation of an environment hostile to regeneration due to the appearance of either a scar tissue (composed of collagen or glia) or axonal growth inhibitors (McConnell & Berry, 1982). Alternatively, the glial proliferation may lead to formation of a growth-supportive environment (Manthorpe et al., 1983; Schwartz et al., 1985; Varon et al., 1983; Williams & Varon, 1983). It appears that the formation of both the hostile and the supportive environments may occur at different time periods after injury (Neumann et al., 1983). It is the net outcome of these opposing contributions which may have an impact on the nature of the response to the injury, i.e, regeneration or degeneration.

Surgical manipulations were performed to determine whether a dense glial scar which is formed by the proliferating glial cells interferes with outgrowth of neurites in regeneration. These studies showed that such a scar does not represent a major obstacle to axonal growth (Reier, 1979; Reier et al., 1983). However, there is no conclusive information as to the contribution of scarring to the failure of regeneration in mammalian CNS (Billingsley & Mandel 1982; Krikorian et al., 1981; Molander et al., 1982).

Nerve transplantation experiments demonstrated that the neuronal environment must be conducive for regeneration (Aguayo et al., 1978; David and Aguayo, 1981; Kao et al., 1977; Richardson et al., 1982). Injured spinal cord and brain axons regenerate readily through grafted columns of Schwann cells in peripheral nerves but seldom enter grafted CNS nerve segments (Aguayo et al., 1978; Kao et al., 1977; Richardson et al., 1982). This indicates that in contrast to glial cells, the non-neu-

ronal cells of the peripheral nerves (e.g., the Schwann cells) or components associated with them (e.g. diffusible or extracellular matrix) have the appropriate properties for regeneration. The contribution of these elements to regeneration is summarized below.

2.1 Diffusible substances and nerve regeneration

Axonal injury causes changes in the activity of diffusible substances originating from the non-neuronal environment and also from the target organ. Thus, for example, the growth of the proximal stump of a transected PNS nerve is facilitated by diffusible proteinaceous molecules possibly anchored in the basal lamina and released from the distal stump of the transected nerve (Longo et al., 1983). Similarly, axonal injury induces increased neurite-promoting activity in extracts of the target organ (Giulian et al., 1986; Henderson et al., 1983; Nieto-Sampedro et al., 1984; Nurcombe et al., 1984). Furthermore, extracts prepared from denervated adult skeletal muscle contain an increased amount of neurotrophic activity which promotes survival of dissociated motor neurons and outgrowth of neurites from explants of spinal cord maintained in serum-free defined media (Nurcombe et al., 1984). This injury-induced increase in activity has also been observed in the brain. For example, the activity of a diffusible substance which is collected from the site of a brain lesion and which affects the survival of chick sensory neurons in culture is increased after lesion (Nieto-Sampedro et al., 1983). Injury-induced increased activity is also manifested by the better survival of brain grafts in wound cavities several days after the injury (Nieto-Sampedro et al., 1983; Nieto-Sampedro et al., 1984).

Injury to optic nerves of fish (Rachailovich & Schwartz 1984) and peripheral nerves of mammals (Skene & Shooter, 1983) both regenerative

systems, causes changes in type and amount of diffusible substances derived from surrounding non-neuronal cells. We hypothesized that among the various polypeptides which show variation in regeneration are components which are involved in glial activation for regeneration. This issue is further discussed in 2.3.

2.2 Extracellular matrix components and nerve regeneration

Extracellular matrices have been shown to have numerous functions in attachment and migration of cells during development and regeneration. Thus, the ability of axons to grow through tissue in vivo during development and regeneration may be regulated by the availability of extracellular matrix components. Among the various components laminin was found to have the most significant effect. Laminin is known to be a key macromolecule in supporting growth and elongation of central and peripheral nervous system neurons both in vivo and in vitro It has been proposed that the continuous expression of laminin by astrocytes is a prerequisite for axonal growth and regeneration in adult CNS (Liesi, 1985; Hopkins et al., 1985). Laminin appears in immature brain cells during CNS development and its presence coincides with phases of neural migration (Liesi, 1985 et al., 1984a,b) In adult brain laminin has been detected only in association with capillary walls and meningeal structures in contrast to its widespread distribution in the peripheral nervous system (McCloon, 1986; Cornbrooks et al., 1983; Bunge et al., 1982). Thus, in vivo adult astrocytes do not ordinarily produce laminin except for short periods, after injury, in some instances (Bernstein et al., 1985; Liesi et al., 1984), but not in all tested nerves (McCloon, 1986). In contrast, astrocytes the of developing rat, like Schwann cells, were shown in primary cultures, to produce and deposit laminin into the extracellular matrix (Liesi et al., 1983).

It appeared that laminin expression is in correlation with growth (Hopkins et al., 1985; Liesi, 1985; Williams & Varon, 1985). Thus, while it is expressed continuously in adult fish optic nerve it is absence in mammalian adult optic nerves. It was therefore tempting to speculate that the absence of laminin along with other extracellular matrix components and cell adhesion molecules in the CNS of mammals is a cause for their poor ability to regenerate, and that this can be circumvented by external stimuli.

2.3 A regeneration like response in a non-regenerative system can be triggered by diffusible substances originating from growing nerves.

We have shown that soluble substances originating from regenerating fish optic nerves, when applied to injured adult rabbit optic nerves, cause manifestations of early events of regeneration in thr latter (Schwartz et al., 1985). The response includes changes in retinal protein synthesis and in growth activity in vitro. Moreover, a similar effect is induced by media conditioned by newborn rabbit optic nerves (Hadani et al., 1984). We therefore proposed the existence of a correlation between ability of nerves to grow and the ability of their environment to provide appropriate factors termed by us as growth associated triggering factors (GATFs; Hadani et al., 1984). These results laid the foundation for our research. The goals which we set for ourselves during the first year of USMRDC support was (i) to determine the extent of the in situ morphological effect induced by these factors (i.e. by the conditioned media of the regenerating fish optic nerves or the newborn rabbit optic nerves, GATFs) (ii) to elucidate the possible biochemical changes induced in the nerves in addition to those already observed in the retinae (iii) to find out the primary target of the applied substances and

thereby to find out their mode of action (iv) to get an insight to their possible biochemical nature.

The major findings that we have obtained towards these goals are summarized below.

2.3.i Growth in GATFs-treated injured nerves.

Morphological studies provided evidence that media conditioned by regenerating fish optic nerve (which from now on will be termed GATFs defined above in 2.3) cause alterations in the injured adult rabbit optic nerve manifested by the prolonged survival of the injured fibers and by the appearance of growth cones (in contrast to their absence in the non-treated nerve) (Lavie et al., 1987). The growth cones were embedded in astrocytic processes. It was therefore suggestive that the glial cells of the treated nerves are probably providing cues needed for axonal growth. This further suggested to us that GATFs include factors which can activate glial cells to acquire growth supportive characteristics.

2.3.ii GATFs include factors which activate glial cells.

In an attempt to find out whether media conditioned by regenerating nerves have the capacity to activate glial cells, we analyzed the neural environment subsequent to the application of GATFs.

Adult rabbit optic nerves were removed and examined immunocytochemically using goat antibodies directed against mouse laminin. Intact nerves and two kinds of injured optic nerves were studied. The latter include nerves that had been crushed and then implanted with a silicone tube containing collagen soaked in fresh medium and nerves that, subsequent to crush, were implanted with a silicone tube containing collagen saturated with GATFs (Solomon et al., 1985). Medium conditioned by regenerating fish optic nerves served as the source of GATFs. This preparation was shown to be free of laminin .

It appeared from our study that the environmental changes caused by GATFs include those which lead to accumulation of laminin (Zak et al., 1987), an extracellular matrix component known to play a role in support of neural growth and elongation (Williams and Varon, 1985). Without application of GATFs laminin immunoreactive sites are not present within the extracellular matrix of the injured adult rabbit optic nerve. The more widespread distribution of laminin resulting from the application of GATFs could be directly induced by the GATFs or could resulted from a cascade of events initiated by the GATFs. It could involve increased production or secretion of laminin or other metabolic changes which affect its accumulation.

Our preliminary results, as well as reports in the literature, reveal that, in intact fish optic nerve (a regenerative system), in contrast to rabbit optic nerve laminin immunoreactive sites are present throughout the nerve matrix mainly in tongues of connective tissue which project in, from the periphery (Liesi, 1985; Hopkins et al., 1985). The continuous presence of laminin in the fish, like other components needed for growth, may be associated with the general plasticity in this system (Bawnik et al., 1986; Quitschke and Schechter, 1984). It therefore appears that the absence of laminin in the matrix of the injured mammalian optic nerve may be a cause, partially circumventable by GATFs, for its poor inherent regenerative ability. Further studies have been carried out in the last year towards the identification of the active substances within the CM responsible for the glial activation; and identification of other components within the CM which may be related to growth.

3. Rationale for the approach taken in this work

In response to injury the adult CNS may exhibit several responses by both the glia and the axons which are needed for regeneration. Nevertheless these neurons rarely accomplish this process with functional recovery.

It is reasonable to believe that either all the needed events are occurring but not at the appropriate time, therefore leading to an abortive process or that one of the key elements in the process are missing and therefore the other needed events don't occur.

Our strategy was to study a system which is endowed with a high regenerative capacity (i.e. the fish visual system), thereby to get an insight to the nature of the problem in the mammalian CNS.

In the visual system of the fish we observed that injury causes environmental alterations. Based on these results we wanted to examine whether the regeneration supportive environment of a regenerative nerve i.e., goldfish optic nerve, can make a neuron of the mammalian CNS (non-regenerative system) to express regeneration-associated response, having in mind that if successful, it would indicate that (a) a non-regenerative system is defective in its environmental factors, and (b) environmental changes in a regenerative system are involved in processes that enable production of factors needed for a regeneration. To carry out such a study, we developed a unique procedure of a transorbital surgery (Solomon et al., 1985) which enabled the use of an adult rabbit's visual system as our experimental model for a non-regenerative system.

At the onset of this study, we transplanted an optic nerve from a carp, which had been conditioned to regenerate by a crush injury, into a transected optic nerve of an adult rabbit by suturing the nerves end to

end. However, immunological rejection was noticed after two weeks. To avoid the rejection, we used a "wrap around" implant with a silicone tube which was internally coated with collagen and contained diffusible substances (i.e. conditioned media) derived from regenerating optic nerves of fish (either carp or goldfish). As a result of this application the rabbit optic nerve and retina showed features which are characteristic of regeneration (Schwartz et al., 1985). These results raised the question as to whether injured optic nerves of adult rabbit have any potential to provide triggering factors. Our result showed that these nerves, do not have the potential. Two possibilities therefore came to mind: either that intact nerves have this potential but lost it as a consequence of injury, or that intact nerves do not have the potential to provide the triggering molecules. In the latter case the potential could have been lost during maturation if produced during development. Our experimental approach of a synthetic "wrap around" implant provided the means to resolve this issue. We were able to show that media conditioned by newborn nerves have a similar activity, while media conditioned by intact or injured adult rabbit optic nerves showed minimal or no activity (Hadani et al., 1984).

It therefore seems that the ability of the axonal environment to provide triggering factors correlates with its growth state (regenerating fish optic nerve and neonatal rabbit optic nerve have the highest activity). This correlation justifies the conclusion that inability of mammalian CNS to produce active factors or to produce them at the appropriate time may be a reason for their poor ability to regenerate.

In an attempt to find out the mechanism underlying the observed effect in the implanted injured rabbit optic nerves we observed that the

glial cells surrounding the injured nerves are also affected. These observations suggested to us that the active conditioned media (derived from actively growing nerves) contain several factors, including neuron specific factor, apolipoproteins and factors which have the capability to activate glial cells. Such activation is manifested by the acquisition of growth features, including production and accumulation of laminin (an extracellular matrix component needed for growth otherwise absent in adult mammalian CNS) and appearance of cell adhesion molecules.

These observations invite the speculation that the glial cells surrounding injured axons of the mammalian CNS like the neurons have the intrinsic ability to develop into regeneration supportive cells if activated properly at the right time. In the absence of such activation these cells will develop into gliosis forming cells which are inhospitable to injured axons growth.

It has therefore been desirable to isolate the regeneration activators; to identify the various components within the conditioned media originating from growing nerves; and to find an optimal way for their administration into non-regenerating nerves. This may lead to a way for intervention to facilitate regrowth after CNS injury by the least invasive method, by making use of the system's own components and modulating them appropriately.

4. Techniques

4.1 Surgical procedures

4.1.1. Carps:

Carps (*Cyprimus carpio*, 800-1200g purchased from Tnuva, Israel) were anesthetised with 0.05% tricaine methansulfonate (Sigma, Israel). Both

optic nerves were then crushed with forceps, taking care to injure the nerve only and leave the surrounding tissue intact. Eight days after the injury, the injured nerves were dissected out and immediately transferred into serum free media (DMEM,GIBCO) for 1.5 h of incubation at room temperature

4.1.2 Rabbit:

Rabbits (albino, The Weizmann Institute, breed Israel) were anesthesized by (5mg/Kg) xylazine and Ketamine (35mg/Kg) administered subcutaneously. The left optic nerve was then exposed using the transorbital surgical approach. The optic nerve was crushed (for 30 sec, with a hemostatic clamp) 4-6 mm distal to the eye globe, and a "wrap around" implant was immediately applied (Solomon et al., 1985). The "wrap around" implant consisted of a silicone tube (2mm and 4mm internal and external diameters, respectively; Burke, FRG), coated inside with collagen (Zyderm, Type V), which was soaked for 24h at 11°C in media containing the active diffusible substances (conditioning media) or fractions obtained after seperations.

4.2 Preparation of conditioned media

The excised fish nerve segments were incubated for 1.5 h in serum free media. At the end of the incubation period the media (300 ul medium/4 nerves) were collected and stored at -20°C..

4.3 Application of horseradish peroxidase

Rabbit optic nerves were crushed and wrapped around with silicone tubes containing media conditioned by optic nerves of neonatal rabbits (Solomon et al., 1985; Schwartz et al., 1985; Hadani et al., 1984). At various stages after this surgical manipulation, horseradish peroxidase (HRP,type VI, Sigma) was applied to a new cut in the optic nerve, made proximal to

the eye, between the optic disc and the primary crush injury. Application of HRP was accomplished by applying surgical sterilized oxidised cellulose (Ethicon) soaked in 30% HRP (in phosphate buffer, pH=7.4). Forty-eight hours after the HRP application, the rabbits were reanesthesized and perfused gravitationally with phosphate buffer (0.1M pH=7.4) through the exposed carotid artery. The whole retinas were then removed and mounted on precoated gelatin slides. The retinal slides were preincubated for 6 hrs in solution containing diaminobenzidine (DAB, 50 mg/100 ml), $CoCl_2$ (0.0025%), nickel ammonium sulphate (0.0002%) and dimethylsulfoxide (1%). At the end of the incubation the slides were transfered for 30 minutes into freshly-made phosphate buffer solution containing DAB (50 mg/100 ml) and covered with permount.

Animals were perfused further with 2% glutaraldehyde in phosphate buffer and the nerves were then excised from the optic disc to the chiasm. The excised nerves were incubated in phosphate buffer containing 30% sucrose. Cryosections (30 μm thickness) were taken and stained for HRP using DAB as the chromophore of choice.

4.4 Immunocytochemical analysis for laminin immunoreactive sites of nerve sections

Normal nerves and those that had been crushed seven days previously were removed and immediately placed into 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.4, for 4 h at 4°C and then transferred into 30% sucrose for overnight incubation at 4°C. Eight micron cryostat sections were placed on gelatinized 8-spot slides (Shandon Scientific) and treated as follows: Sections were exposed to PBS containing 1% BSA (for 3 h at room temperature or overnight at 4°C) and then with 0.15M Tris-HCl, 0.1M glycine, pH 7.2, (45 min at room temperature) to block non-specific

binding of antibody and autofluroescence due to fixation. Serum of sheep containing antibodies against murine EHS sarcoma laminin (diluted 1:1000, gift of Dr. H. Kleinman, NIH) or PBS containing 0.2mg/ml BSA were then applied for 2 h at room temperature. Sections were washed (4x, 10 min each) in PBS containing 0.2 mg/ml BSA and then exposed to fluorescein isothiocyanate-conjugated rabbit $F(ab')_2$ anti-goat IgG (Cappel Laboratories, Penn., 20 ug/ml, also reacts with sheep IgG) diluted 1:100 for an additional 2 h at room temperature. The sections were mounted in Gelvatol 20-30 (Monsanto Corp., N.Y.) in tris-HCl, pH 8.2. Fluorescence was examined with a Zeiss Photomicroscope III using a 100 watt mercury lamp and Neofluar 25x objective. All fluorescent micrographs were taken using 1 min exposures on Kodak Tri-X film and were developed identically.

4.5 In vitro approach for laminin production

4.5.1 Treatment of C-6 cells

C-6 cells were grown in wells of microtiter plates in Dulbecco modified Eagles medium (DMEM) containing 10% fetal calf serum (FCS). When the cells reached confluency the medium was changed to Waymouth's medium supplemented with insulin (5 μg/ml), bovine serum albumin (BSA, free of fatty acids, 0.5 mg/ml) and antibiotics (streptomycin/penicillin, 100μg/ml). The cells were kept in this medium for 24 h, followed by an additional 24 h period in the same medium supplemented with media conditioned by the regenerating fish optic nerves. The cells were then examined for thymidine incorporation and laminin appearance.

4.5.2 ELISA screening methods for surface antigens

Cells which were grown in microtiter plates, were centrifuged 3 min at 1000xg and then washed with phosphate buffered saline (PBS). Nonspecific sites were blocked by BSA for 30 min at room temperature.

Following this incubation the supernatant was collected and antibodies at the appropriate dilutions were applied for 30 min. The cells were then washed with PBS and treated with the second antibodies conjugated to horseradish peroxidase (50 µl were added to each well for 1-3 h incubation at 37°C or overnight at 4°C). Cells were washed, and 100 µl of substrate was added to each well. Reaction was stopped by the addition of sodium azide (0.01 w/v) and citric acid (0.1M) Absorbance was recorded at 410 nm.

4.6 Two dimensional gel electrophoresis

Samples for the first dimension of isoelectric focusing were prepared in denaturing solution containing 9.5 M urea, 2% NP-40 (nonidet P-40), 2% ampholines (1.6% pH 5 to 7, 0.4% pH 3.5 to 10), and 5% 2-mercaptoethanol. The second dimension was performed on a slab gel containing 15% acrylamide (O'Farell, 1975). After electrophoresis in the second dimension, the gels were fixed and stained.

4.7 Immunoblot

The tested samples were separated by one or two dimensional gel electrophoresis (SDS-PAGE 15% polyacrylamide). On each slot of the gel, samples corresponding to 50 µg of protein were applied. Following electrophoresis the proteins were blotted immediately onto nitrocellulose (0.45 µm; Schleicher & Schuell, Germany) for 2 h, 200 mA in transfer buffer (15.6 mM Tris, 120 mM glycine, pH = 8.3; Anderson et al., 1982). The blotted nitrocellulose was incubated overnight at room temperature in phosphate buffer containing 0.3% Tween-20 (polyoxyethalenesorbitan monolaurate, Sigma). The immunological reaction was carried out at room temperature, sequentially: incubation for 1 h with the primary antibodies diluted 1:100, wash, incubation for 1 h with Horseradish peroxidase

(HRP) conjugated to protein-A (diluted 1:1000) wash, and incubation with 4-chloro-1-naphthol until an optimal color had developed.

4.8 Solid phase radioimmunoassay

Wells of flexible microtiter plates were coated with CM derived from intact fish optic nerves and injured nerves excised one and eight days after injury (250 ng protein in 50µl phosphate buffer). After overnight incubation the wells were washed with phosphate buffer containing 1% BSA and further incubated with anti-apo-A-I antibodies at various dilutions (1:200-1:50000). After 2 h of incubation the wells were washed, and further incubated with ^{125}I-goat anti rabbit Ig (100.000 cpm/well) for an additional 2 h. At the end of the last incubation the wells were washed, dried and counted.

4.9 Immunocytochemical localization at the electron microscope level

4.9.1 Fixation.

For the studies at the light microscope level optic nerves were dissected out, cleaned, and then incubated in paraformaldehyde (4%) in PBS, pH7.4 for 2 hours at 4°C, after which they were transfered into sucrose solution (30%) for overnight incubation at 4°C. Cryosections (10 µm thickness) were placed on gelatin coated slides and treated as follows: sections were incubated in PBS containing 1% BSA for 30 min at room temperature and then in solution containing 0,15 M Tris-HCl, 0,1 M glycine, pH7,2 for 20 minutes at room temperature in order to block non specific binding of antibody and background fluorescence due to fixation. Primary antibodies after affinity purification were incubated for 2 hours at room temperature. Sections were washed (4x7 min each) in PBS and then exposed for 2 hrs to a fluorescein isothiocyanate conjugated goat anti rabbit IgG (Cappel laboratories, Pennsylvania) followed by wash (4 x 7 min each).

Fluorescence was examined with a Zeiss photomicroscope III using a 100W lamp and neofluar 16x objective. All fluorescent micrographs were taken using 1 min exposures on Kodak Tri-x-film and were developed identically.

4.9.2 Electron Microscopy.

Optic nerves were dissected out, cleaned and then kept in 2% glutaraldehyde 1% paraformadehyde in cacodylate buffer 0,1M. Following the fixation, the preparation was chopped in 1mm segments, placed back in the fixative, and postfixed with osmium tetroxide, in cacodylate buffer. The preparation was then stained in uranylacetate (2%) in water for 30 seconds and dehydrated in ethanol in increasing concentrations of propylene oxide. Embedding was done in Epon. Polymerization was performed at 60°C for three days. Thick sections (1-3μM) were prepared using LKB II ultratom. These sections were stained with toluidine blue (1%). Thin sections (500-700 A°) were collected on Copper grids. The grids were treated with H_2O_2 (10%) for 10 min at room temperature, then washed in water. The washed grids were then incubated in a PBS solution containing ovalbumin (1%), Tween-20 (0,05%) for 15 min to block nonspecific binding. Grids were then treated with the first antibody (affinity purified rabbit anti APO-AI antibodies) for 2 hours and then exposed to the second antibody which was goat anti rabbit IgG tagged with colloidal gold particles (Janssen, Belgium), then washed again, and stained with uranylacetate saturated in ethanol (50%) and for 4 min in lead buffer. The sections were visualized in a Phillips 410 electron microscope.

5. Summary of the major results and achievements during the year 1986-1987

5.1 Purification and characterization of a low molecular weight GATF

5.1.a Purification

We have shown as was summarized in section 3 that media conditioned by regenerating fish optic nerves when applied to injured optic nerves of adult rabbit causes biochemical changes in the retina and environmental changes in the nerves. Our initial attempt for identifying the active component has used the *in vivo* effect of the conditioned media as the bioassay of choice. Specially, the ability of the conditioned media, or fractions derived for it, to cause biochemical changes in the retina was used as our bioassay. Using this bioassay we have succeeded to purify a low molecular weight substance (less than 500 dalton). Such a molecule when applied to injured optic nerve of adult rabbit caused a general increase in protein synthesis and selective increase of a few polypeptides, putative growth associated proteins (Harel, A., Rubinstein, M., Solomon, A.,Belkin, M. and Schwartz, M., manuscript in preparation).

The purification scheme used to accomplish this task is summarized below.

conditioned medium
132 optic nerves
10 ml, 3.3 mg glycine equivalents

↓ sephadex G-25
fractions 81-120, 160 ml
↓ lyophilization
redissolve in water 1.8 ml, 21 ug glycine equivalents

↓ RP-HPLC
fractions 33-34 2 ml 3.1 μg glycine equivalents

Conditioned medium of injured carp optic nerves (10 ml) was spun (1000xg, 2 min) and the supernatant was filtered through a 0.45 μm filter (Schleicher & Schuell, W. Germany). The filtrate was then applied to a Sephadex G-25 coarse (Pharmacia, Sweden) column (2.5x50 cm equilibrated with 50 mM NaCl and 5 mM phosphate buffer, pH 7.4). Elution was performed with 50 mM NaCl and 5 mM phosphate buffer pH 7.4 at a flow rate of 96 ml/hr and fractions of 4 ml were collected. the eluate was monitored continuously by u.v. absorbtion at 280 nm. The elution profile is shown in Fig. 1B. In a control experiment conditioned medium of uninjured carp optic nerves (8 ml) was fractionated on the same sephadex G-25 column under the same conditions (Fig. 1A). The content of fluorescamine positive material was determined and was experessed in glycine equivalents.

Fractions 81-120 from the Sephadex G-25 chromatography were pooled, lyophilized and redissolved in distilled water (2 ml). Acetic acid 0.12 ml was added, the solution was spun (10,000xg, 2 min) and the supernatant was applied to an octodecylsilane silica HPLC column (ultrashpere ODS, 5 μm 4.6 x 250 mm, equilibrated with 0.3% aqueous trifluoroacetic acid, Beckmann Instruments USA). The column was washed with 10 ml of 0.3% trifluoroacetic acid (Buffer A) at a flow rate of 0.5 ml/min and elution was then performed at 0.5 ml/min by a linear gradient of acetonitrile (0 to 10% during 60 min) followed by a gradient (10 to 50% during 10 min) in Buffer A. Fractions of 1 ml were collected. Protein was monitored by a fluorescamine monitoring system. The amount of fluorescamine reactive material was determined in each fraction using glycine as a standard. The elution profile is shown in Fig. 2.

5.1.b Characterization of the purified GATF.

Bioassay: Conditioned media or recovered fractions were applied to injured optic nerve of adult rabbit as described in section 4.1.2 All the rabbits, which were implanted either with the crude conditioned media or with the various recovered fractions, were sacrified one week after the surgery. The retinae on both sides were then separately excised and were processed for determination of total incorporation of [^{35}S]methionine or for profile of specific polypeptides by gel electrophoresis as was described previously (Hadani et al., 1984; Schwartz et al., 1985). The ratio L/R represents the specific activity of labeled protein in the retina of the treated (injurec only or injured and implanted with conditioned media or recovered fractions) relative to the contralateral, non-injured, non-treated side. Each retinal preparation was then tested for protein profile by electrophoresis on acrylamide gels. For visualization of the labeled proteins the gels were treated with DMSO PPO and fluorographed using Agfa Currix RP2 film.

Chromatography of the purified GATF, on ultrasphere ODS reversed phase column under the same conditions used for isolation gave a single peak of naturally fluroescent substance in fractions 33-34, corresponding to 8% acetonitrile.

The low molecular weight substance did not reproduce the full effect of the original conditioned media on the nerve. Therefore a search was made to identify additional components such as the one responsible for the environmental activation.

5.2 The crude GATFs contain a distinct component(s) which can directly activate glial cells

We have shown in the previous report that CM of regenerating fish optic nerve when applied to injured adult rabbit optic nerve cause the increased distribution of laminin immunoreactive sites (Zak et al., 1987). In an attempt to find out whether such CM contain component(s) which can directly activate glial cells we established in vitro conditions. Specifically we have established the C-6 glioma cells line for use in our bioassay as described in section 4.1.5

The cells were kept in the medium containing BSA and insulin for 24 h and then for an additional 24 h in the same medium supplemented with the crude GATFs (media conditioned by the regenerating fish optic nerves). Twenty four hours later the cells were examined for laminin immunoreactive sites either by the ELISA screening method for surface antigen or by immunocytochemistry.

Laminin immunoreactivity could be detected normally in cells of C-6 glioma cell line. The level of this immunoreactivity was elevated in cells treated with media conditioned (CM) by regenerating fish optic nerves. The optimal effect was observed at concentration of 0.1 μg/ml. Higher concentrations did not have any further effect or were even inhibitory suggesting the possible existence of inhibitory elements . CM-stimulated effect was confirmed by immunofluoresce staining . The immunoreactivity was most intense in cells treated with media conditioned by regenerating fish optic nerves at a concentration of 0.1 μg/ml protein. (Fig. 3).

The identity of the laminin immunoreactive protein in C-6 glioma cells was verified by the use of heparin affinity column followed by

immunoprecipitation and gel-electrophoresis. Fig. 4 shows the results of analysis of C-6 glioma cells metabolically labelled with [^{35}S]methionine. The extracellular matrix components were solubilized by high salt (1M NaCl) and were further used for immunoprecipitation. Analysis by gel electrophoresis of the immunoprecipitate revealed the presence two polypeptides corresponding to the two polypeptides of laminin migrating slightly faster. Preliminary results showed that the factor responsible for activation for the laminin production by the glial cells is heat sensitive, and early fractionation studies revealed that its molecular weight is between 10kDa and 30kDa (Cohen, A. and Schwartz , M. manuscript in preparation).

Currently we have done the first step towards the isolation of the components within the CM responsible for the direct activation of the glial cells. This procedure involved gel filtration and separation on ion exchange column by FPLC. Results will be reported in the first quarterly report of the third year of this support.

5.3 GATFs contain apolipoprotein

We have previously observed that media conditioned by regenerating fish optic nerve show elevation in a 28 kDa polypeptide (Rachailovich & Schwartz, 1984). Under similar experimental conditions an elevaiton in a 37 kDa polypeptide was observed in media conditioned by regenerating peripheral nerves of mammals (Skene & Shooter, 1983; Muller et al., 1984). The 37 kDa polypeptide in regenerating peripheral nerves of mammals was identified as apolipoproteins-E (apo-E) (Ignatius et al., 1986; Snipes et al., 1986). Similarly the 28 kDa polypeptide in the fish CNS was also identified as apolipoprotein but a-I rather than E. We have achieved the identification of the 28 kDa polypeptide by isolating apoli-

poproteins from the fish plasma. The fish plasma contained apo-A-I but not apo-E (Fig. 5). Antibodies were raised against the fish plasma apo-A-I and were further used for the identificaiton of the 28 kDa in the CM.

It appeared that media conditioned by the regenerating fish optic nerves contain 28 kDa polypeptides which cross-reacted immunologically with the antibodies directed against fish plasma apo-A-I (Fig. 6).

The level of the accumulated apo-A-I in the regenerating fish optic nerves was found to be about 35% higher than in non-injured fish optic nerves (Table 1). In sections taken from fish optic nerves apo-A-I immunoreactive sites were found in both non-injured and injured fish optic nerves. However, higher labeling was found in injured nerves already one day after injury. The increase labeling was more pronounced in the nerve segment between the site of the injury and the chiasm (Fig 7). Ultrastractural localization of apo-A-I immunoreactivity in the fish optic nerves revealed labeling in astrocytes and macrophages. Visualization was achieve by using second antibodies tagged with gold particles (Fig. 8,9). It appeared that labeling in macrophages was higher than in astrocytess (Table 2). Within the macrophages it appeared that gold particles were more abundant in granules than in cytoplasmic area surrounding these granules (Table 2), (Hernandez, M., Harel, A., Fainard, M. and Schwartz, M., manuscript in preparation).

Little is known about the role of apolipoproteins in the nervous system. Apo-E, for example, was suggested to be involved in the mobilization and reutilizaiton of lipid in growth during development and repair. This suggestion is supported by the finding of apo-E associated with astrocytic glia in the central nervous system (Boyles et al., 1985).

Brain cells in culture secrete apo-E and metabolize apo-E containing liposomes (Boyles et al., 1985; Pitas et al., 1985). The astocytes possess apolipoprotein B,E, (LDL) receptors capable of binding and internalizing apoipoprotein-E containing lipoproteins. It was therefore suggested that apo-E may function to redistribute lipid (Pitas et al., 1987). In regenerating peripheral nerve, apo-E - associated lipid particles released from segments of the injured nerves were shown to be delivered to neurites and their growth cones for membrane biosynthesis (Ignatius et al., 1987).

Apo-E in peripheral nervous system, a regenerating system, is originating from macrophages and to a limited extent from non-myelinating Schwann cells. It has been proposed that Schwann cells and macrophages in the PNS and glial cells in CNS play a common role during the new growth and/or maturation. Upon injury, macrophages in PNS infiltrate the distal stump and express large amount of Apo-E, whereas in CNS the expression of apo-E, which is ordinarily by astrocytes, is terminated (Stoll & Muller, 1986). It may be possible that the fish CNS, expressing apo-A-I, can be differentiated from CNS of higher vertebrates by being able to provide apo-A-I, after injury both by astrocytes and infiltrating macrophages.

5.4 GATFs combined with dditional treatment modalities

We have previously shown that when GATFs (originating from regenerating fish optic nerves) are applied to injured optic nerve axons of adult rabbit they cause a regeneration-like changes in both the neurons and their environment (Schwartz et al., 1985; Stein-Izsak et al., 1986; Lavie et al., 1987; Zak et al., 1987) As was discussed in Chapter 5.2 we attributed the observed effect to the activation of glial cells which

thereby became conducive rather than inhospitable to growth (Zak et al., 1987). The possibility that the growth dependes on the addetive or even synergistic effect of the two already identified factors (i.e. the low molecular weight molecule and the glial modulating factors) and possible other components within the CM has to be examined. The growth, however, is still limited (Levie et al., 1987).

We considered the possibility that by combining this treatment with one that prolongs the survival of the traumatized nerves such as by implanting in addition fibroblast growth factor (FGF) or delays and attenuates scar formation (by the glial cells) such as low energy laser irradiation (Rochkind et al., 1986; Schwartz et al., 1987; Rochkind et al., 1987), we may achieve a better effect on the nature of the environment and make it more conducive to growth.

Preliminary results suggest that indeed the combined treatments result in abundant growth of non-myelinated fibers within the rabbit optic nerve into the distal stump. A special emphsis should be made that this approach leads to a growth within the native CNS environmen and therefore has the potention of leading to appropriate reconnection as compared with other currently known approaches which involve environme ntal substitution.

6. Conclusions

In the second year of our support from the US Army Medical Research and Development Command we were able to substantiate our observation from the first year of our support and to further extend them. Our achievements are summarized below:

1) Media conditioned by regenerating fish optic nerves (representing growing nerves) which was designated by us as growth associated triggerign factors (GATFs) contain (i) a small molecular weight substances which when applied to injured optic nerves of adult rabbit cause changes in the protein synthesis of the corresponding retinas and (ii) and a glial modulating factor which when applied the glial cells in culture (C-6 glioma) causes elevation in accumulation (revealed by ELISA and by immunocytochemistry) and elevation in metabolically labeled laminin (revealed by immunoprecipitation). These factors are currently purified and hopefully will provide means for modulating glial cell response to injury and thereby reach conducive conditions for growth.

2) Media conditioned by regenerating fish optic nerves was found to contain several growth-related components. One of which was identified as apolipoprotein-A-I. A 28 kDa was identified as apo-A-I and is suggested to be the mammalian counterpart of a 37 kDa polypeptide identified as apolipoprotein-E. Apo-A-I levels were shown to be increased after injury and in the nerves were localized in macrophages and actrocytes. It is suggestive that apo-A-I is involved in scavanging myelin degradation products to pave the way for regeneration and for the reuse of these lipid product upon building of new fibers and regrowth.

3) Preliminary results (not shown in this report) suggest that application of GATFs to injured nerves which are also treated with growth modality showed to cause delayed degeneration (low energy laser) cause extensive growth of non-myelinated fibers across the injury within scaffolding created by astrocytic processes. The events underlaying

the changes leading to this abundant growth within such modulated CNS environment is currently investigated.

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Legend to Figures

Figure 1: Steric exclusion chromatography of conditioned media (CM). CM of normal (A) and injured (B) carp optic nerves (10 ml) were applied to a sephadex G-25 coarse (2.5x50 cm) pre equilibrated with 50 mM NaCl and 5 mM phosphate buffer, pH 7.4. Elution was performed with the same buffer at a flow rate of 96 ml/hr. The effluent was monitored by u.v. absorbtion at 280 nm and fractions of 4 ml were collected. The column was calibrated with vitamin B_{12} and Tryptophane as molecular weight markers. The major activity was obtained in fractions 81-120.

Figure 2: Reversed-phase HPLC. Combined and lyophilized fractions 81-180 from the steric exclusion chromatography (Fig. 1B) were dissolved in aq acetic acid (1M 2 ml) and applied to an octodecyl silica HPLC column (ultrosphere ODS 5 μm 4.6x250 mm Beckmann Instruments). The column was preequilibrated in 0.3% aq trifluoroacetic acid and elution was done at a flow rate of 0.5 ml/min by a linear gradient of acetonitrite (0% to 10% during 60 min) followed by a step of 10% to 50% during 10 min. Fractions of 1 ml were collected. The effluent was monitored by post column reaction with fluorescamine which consumed 1-2% of the column effluent. Biological activity was found in fractions 33 and 34.

Figure 3 Laminin immunofluorescent sites in cells of C-6 glioma cell line.

Cells were grown on glass coverslips in DMEM containing 10% FCS till they reached confluency. The cells were then washed and incubated for 24 h in Waymouth medium supplemented with insulin and bovine serum albumin. After twenty-four hours the various plates were further treated with CM or with FCS in the supplemented Waymouth medium. After additional 24 h

the medium was collected, cells were washed (X3) in tris buffer (10 mM tris 7.4, 150 mM NaCl) and then fixed for 15 min in ethanol (96%) followed by wash in tris buffer. Reaction with the primary antibodies was carried out for 45 min at 37°C, followed by wash (X3) in tris buffer and then for 45 min in FITC conjugated to goat anti Rabbit IgG (1:100) followed by 3 washes in tris buffer. Samples were then coated with solution of glycerol (90% in PBS) and covered with glass.

A. cells treated with 1 μg/ml CM; B. cells treated with 0.1 μg/ml CM; C. Cells treated with 10% FC; D. cells kept in Waymouth free of serum or conditioned medium. All groups were staining with anti-laminin antibodies. Photograph shown in E represent cells in A incubated with rabbit preimmune sera rather than with the anti-laminin antibodies.

Figure 4 Identification of laminin in media of C-6 cells by heparin affinity column and immunoprecipitation.

Cells were treated as in Figure 1. The last 24 h of incubation was carried out in medium which was supplemented also with [^{35}S]methionine (12 μCi; 113 μci/m mole, Amersham). Laminin containing fraction was extracted from the cells by high salt [1 M NaCl] in 50 mM Tris pH 7.4 and was further immunoprecipitated using antibodies specific to laminin (BRL). The binding was carried out in solution containing 0.5% Np-40 and 0.1% SDS for 2h at room temperature. The formed complexes were precipitated by the addition of 10% fixed staphlococcus aureus suspended in buffer containing 140 mM NaCl, 10 mM Tris pH 7.4, 5 mM EDTA and 0.5% Np-40, followed by centrifugation at 2000xg. The pellet was collected, washed and finally suspended in 75 μl electrophoresis sample buffer, heated to 100°C and centrifuged at 15,000xg for 3 min and electrophoresed in SDS-PAGE (5% acrylamide with 3.5% stacking gel, Ref).

Labeled molecular gel markers were electrophoresed in these conditions. Note the appearance of a 210 kDa polypeptide which migrated very close to the non-labeled purified laminin obtained from mouse EHS sarcoma. The 400 kDa may represent the high molecular polypeptide of laminin. The additional coprecipitated polypeptide, the 220 kDa, may be fibronectin.

Figure 5: Analysis of carp plasma lipoproteins by SDS-PAGE. The lipoproteins having the densities mentioned on top were dialysed, delipidated, solubilized and applied to the gels (75 μg protein per lane) Human albumin (10 μg/lane), nonfasting human plasma lipoprotein of d < 1.006 g/ml (75 μg/lane) and human HDL (75 μg/lane) were also electrophoresed for comparison. Apoprotein migration was identified from migration of purified apoproteins and molecular weight markers (not shown).

Figure 6: Two-dimensional gel electrophoretic analysis of media conditioned by regenerating fish optic nerves and the corresponding Western blot using antibodies directed to Apo-A-I. Samples The second dimension was carried out on a slab gel containing 15% acrylamide. After the run the gels were fixed, stained and photograped (A). Note the appearance of three spots corresponding to 28 kDa molecular weight. The corresponding Western blot is shown in B. Samples for the first and second dimensions were prepared and electrophoresed as described above. After the second dimension the gel was blotted separately onto nitrocellulose. The subsequent manipulations were performed as described in the legend to Fig. 5. Note the appearance of three spots in the 28 kDa molecular weight apo-A-I recognized by the antibodies.

Figure 7: Localization of apo-A-I immunoreactive sites in injured fish optic nerves. Cryosections (20 μm) were taken from injured fish optic nerves (dissected out 8 days after injury). The nerves were separated into two segments, between the optic disk and the site of injury; and between the site of injury and optic chiasm. Higher labeling is seen in the site of injury and the distal segment than in the segment which is proximal to the optic disk x360.

Figure 8: Apo-A-I immunoreactive sites in macrophages of fish optic nerves. Nerves were dissected out and fixed in phosphated buffer containing paraformaldehyde and glutaraldehyde. The nerves were embedded in polybed and then thin sections were taken. The sections were treated with affinity purified antibodies directed to Apo-A-I followed by second antibodies conjugated to gold coloidal particles (5-15μm). Micrograph A shows macrophage stained with the specific antibodies whereas micrograph B shows macrophage of control section treated only with the secohd antibodies. Micrograph C is an enlargement of area in A showing that the gold particles are selectively concentrated in granuls more than in surrounding cytoplasmic area. A, B x 15470°C x 28900.

Figure 9: Apo-A-I immunoreactive sites in fish optic nerves astrocytes. Nerves were treated as is described in the legend to Figure 2. Micrograph A shows astrocytes stained with the specific Apo-A-I affinity purified antibodies whereas, B shows control astrocytes treated only with the second antibodies. Micrograph C shows an enlargement of a chosen area in B. A,B x 15470: C x 33670.

Table 1: Accumulation of apo-A-I in media conditioned by fish optic nerves after injury.

	mean ± S.D. (o.D. 504 nm)		
non-injured[a]	PC-1[b]	PC-8[c]	PC-30[d]
510.33 ± 86.6	690.67 ± 83.5	648 ± 119.7	628.8 ± 54.9

- Group a is different from group b by student 2-tailed t-test with $p<0.005$.
- Group c is different from a with $p<0.05$.
- Group d is different from a with $p<0.5$.
- Group b and c are different with $p<0.2$. injury.

Table 2:

Gold particles per 1.5 cm_2 area. Mean ± SD.			
Macrophages	Astrocytes	Macrophages	
		granules	cytoplasma
n=10		n=15	
12.2 ± 5.4	6.5 ± 3.5	4.71 ± 2.8	0.6 ± 0.9
p<0.01			P<0.005

Immunocytochemoical was carried out as described in the legend to Figure 3 and 4. Pictures were taken and printed. The number of gold particles was counted at a picture magnification of 15470 for the astrocytes and the macrophages and of 28900 for the destinction between macrophages granules and cytoplasm. Statistical analyses is based on student two-tailed t-test.

Fig. 1

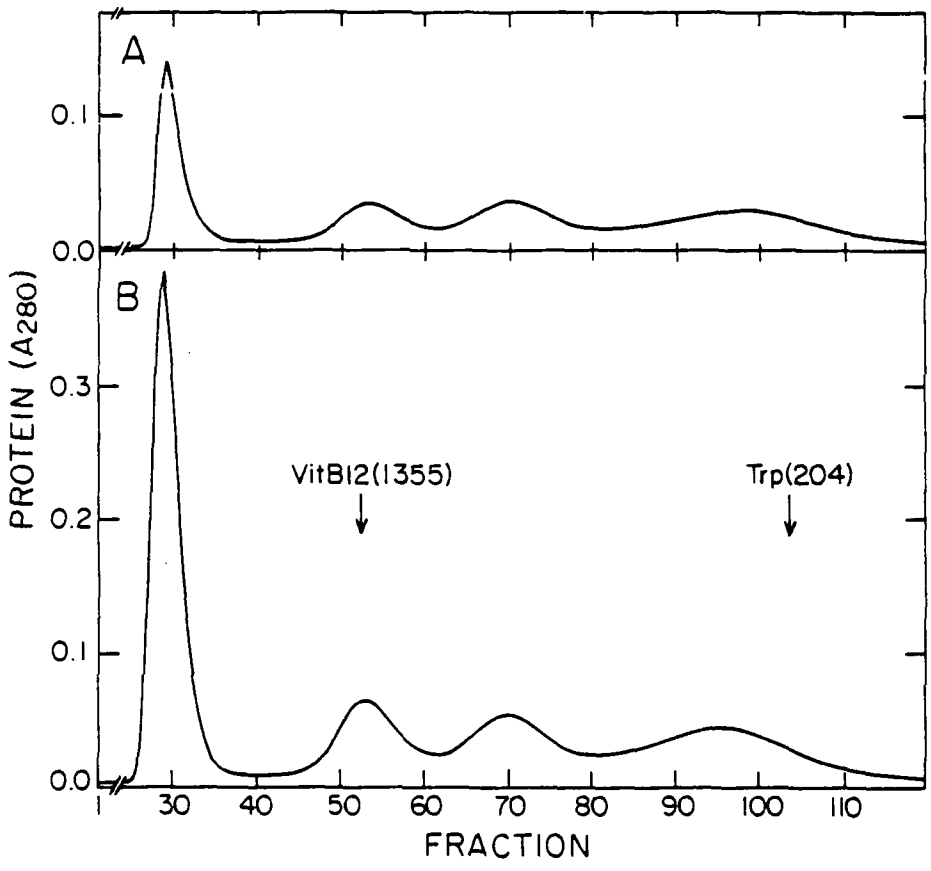

A
B
0.1
0.0
0.3
0.2
0.1
0.0
PROTEIN (A_{280})
VitB12(1355)
Trp(204)
1
30
40
50
60
70
80
90
100
110
FRACTION

Fig. 2

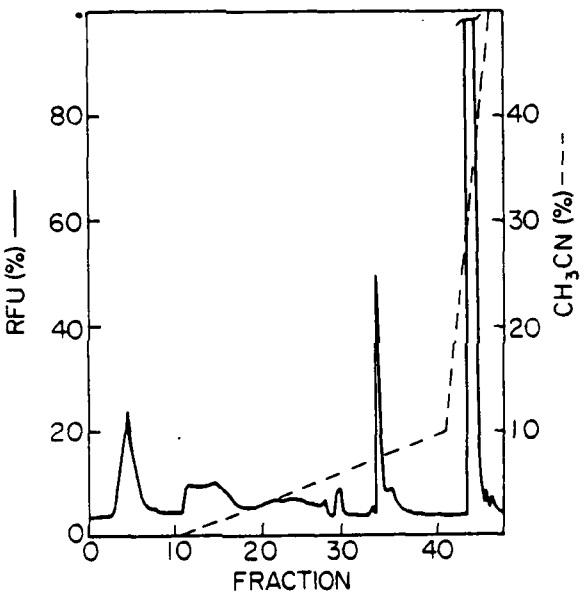


Fig. 3

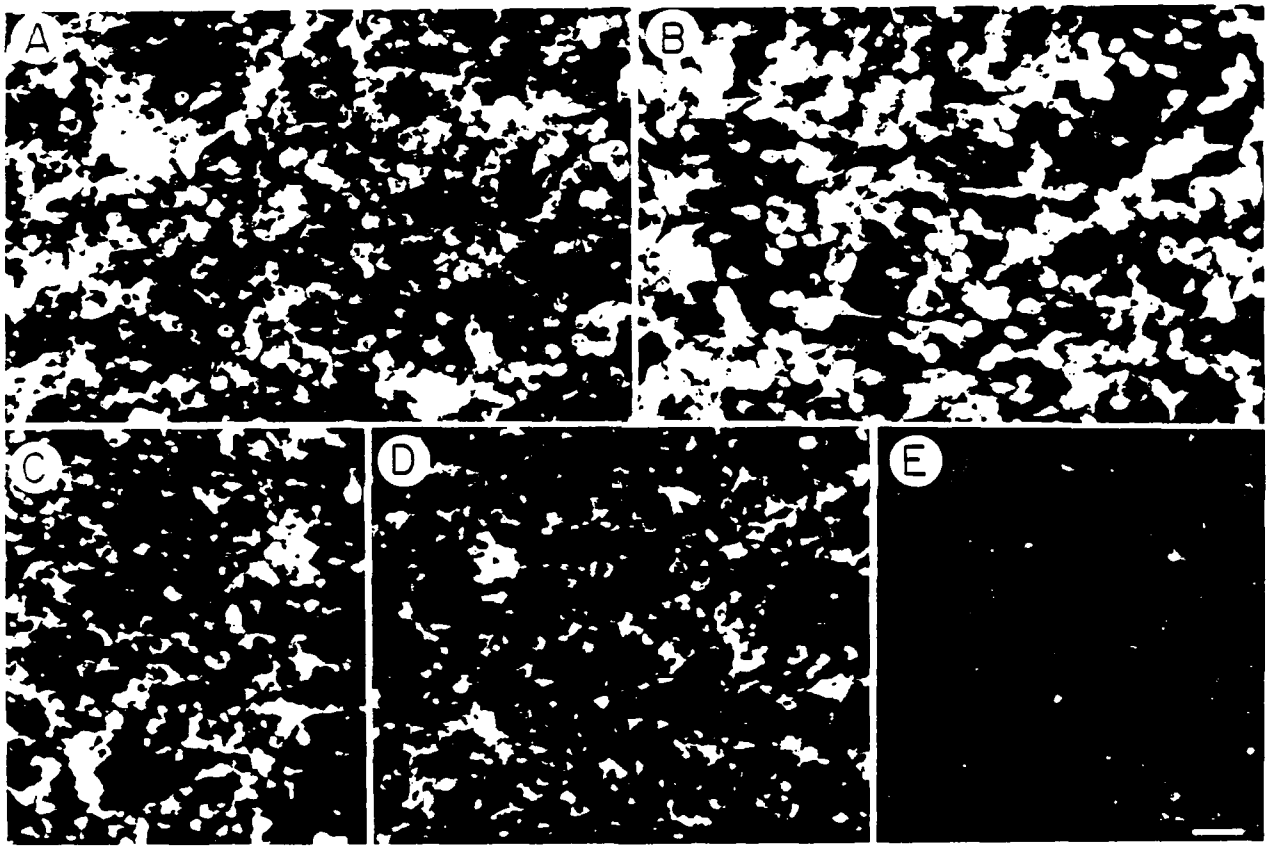


Fig 4

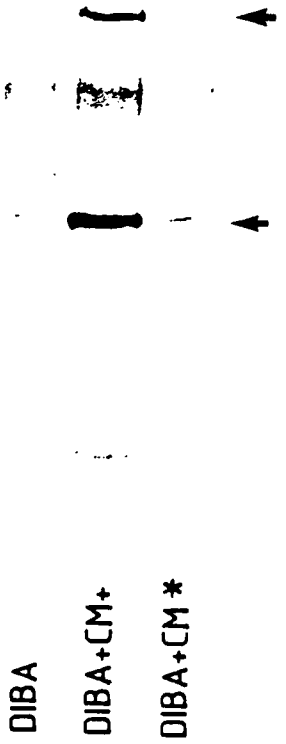


Fig. 5

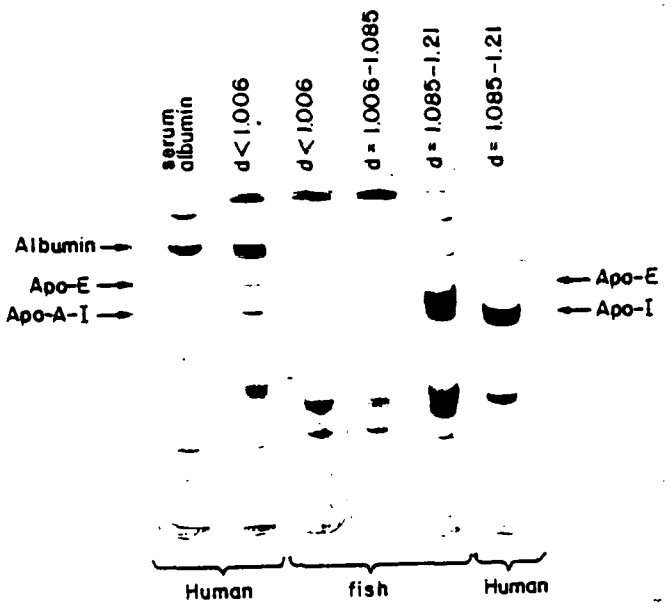

serum albumin
d < 1.006
d < 1.006
d = 1.006-1.085
d = 1.085-1.21
d = 1.085-1.21
Albumin
Apo-E
Apo-A-I
Apo-E
Apo-I
Human
fish
Human

Fig. 6

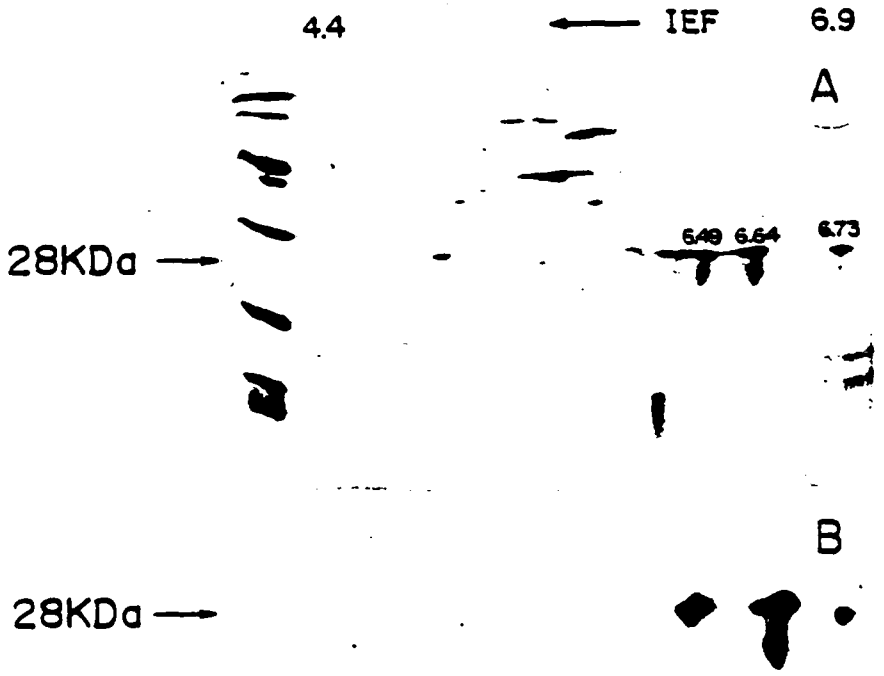


Fig. 8

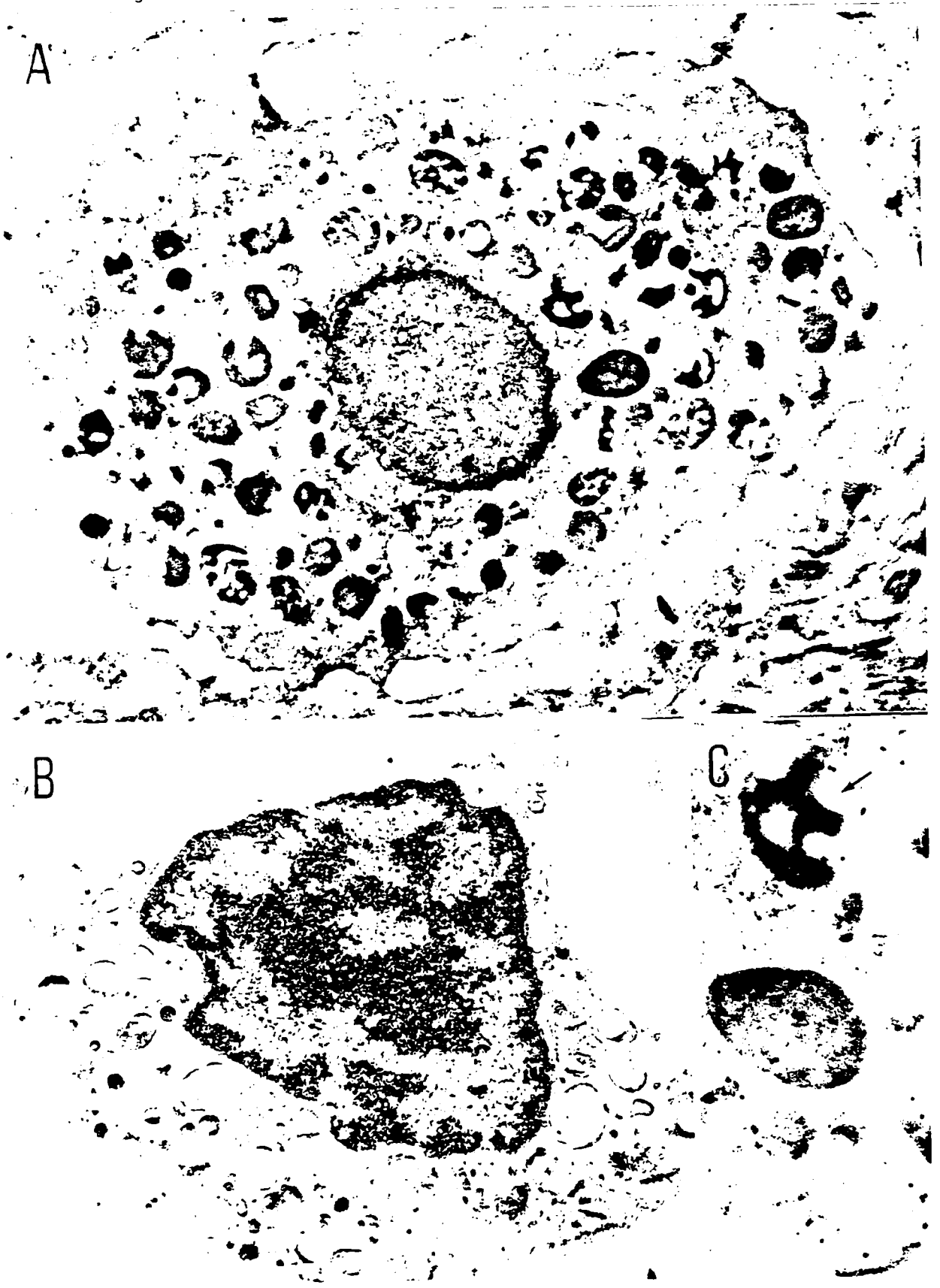


Fig. 7

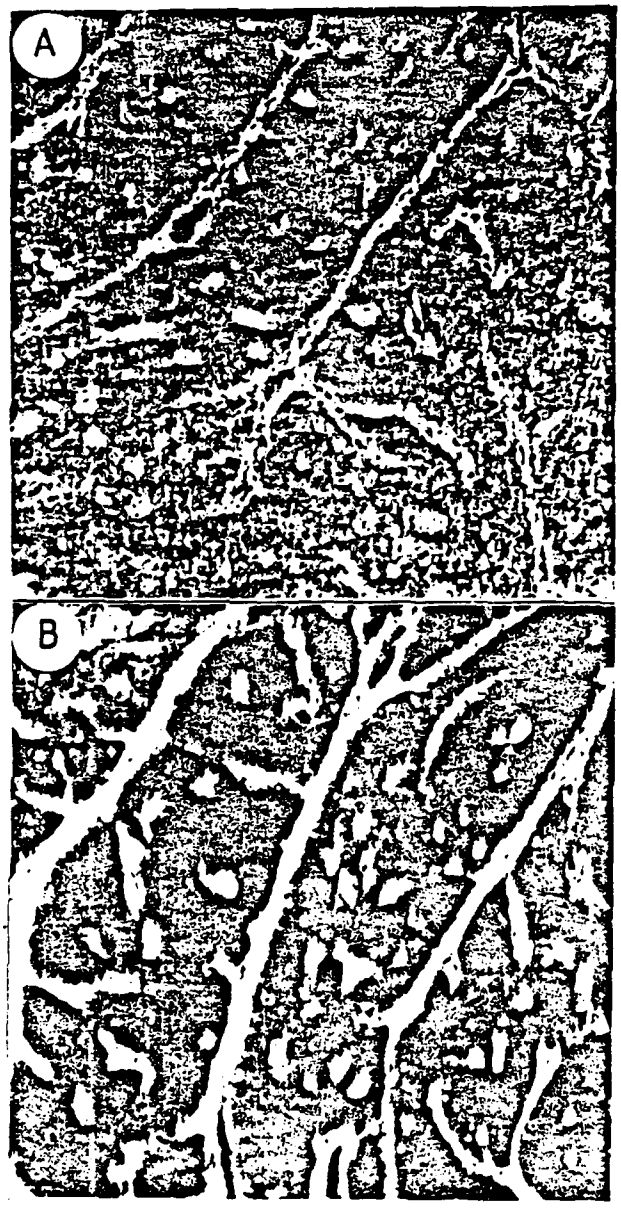
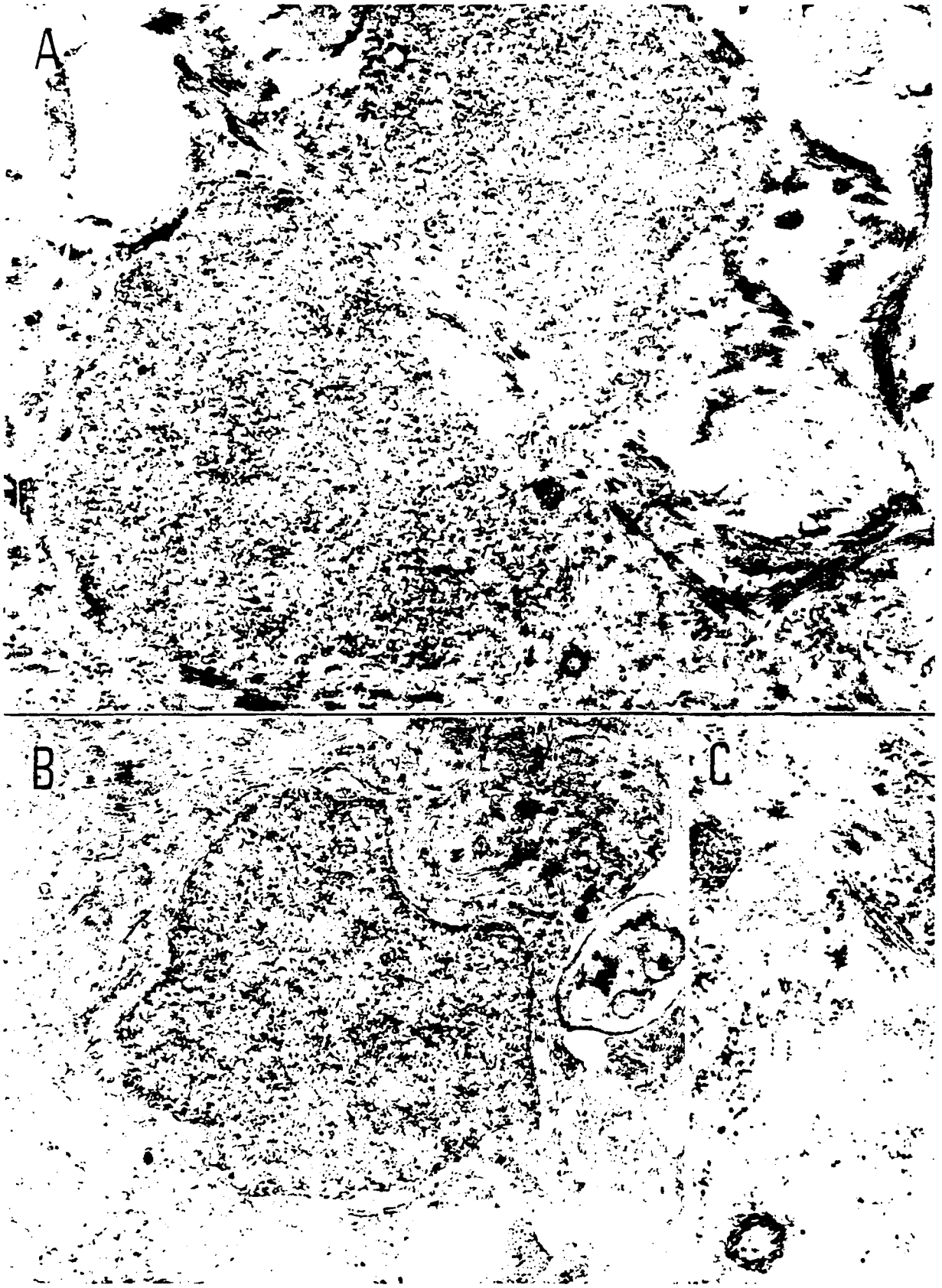


Fig. 9



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